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A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids

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A cinnamoyl esterase, ferulic acid esterase A, from *Aspergillus niger* releases ferulic acid and 5-5- and 8-*O*-4-dehydrodiferulic acids from plant cell walls. The breakage of one or both ester bonds from dehydrodimer cross-links between plant cell wall polymers is essential for optimal action of carbohydrases on these substrates, but it is not known if cinnamoyl esterases can break these cross-links by cleaving one of the ester linkages which would not release the free dimer. It is difficult to determine the mechanism of the reaction on complex substrates, and so we have examined the catalytic properties of ferulic acid esterase A from *Aspergillus niger* using a range of synthetic ethyl esterified dehydrodimers (5-5-, 8-5-benzofuran and 8-*O*-4-) and two 5-5-diferulate oligosaccharides. Our results show that the esterase is able to cleave the three major dehydrodiferulate cross-links present in plant cell walls. The enzyme is highly specific at hydrolysing the 5-5- and the 8-5-benzofuran diferulates but the 8-*O*-4-is a poorer substrate. The hydrolysis of dehydrodiferulates to free acids occurs in two discrete steps, one involving dissociation of a monoesterified intermediate which is negatively charged at the pH of the reaction. Although ferulic acid esterase A was able to release monoesters as products of reactions with all three forms of diesters, only the 5-5- and the 8-*O*-4-monoesters were substrates for the enzyme, forming the corresponding free diferulic acids. The esterase cannot hydrolyse the second ester bond from the 8-5-benzofuran monoester and therefore, ferulic acid esterase A does not form 8-5-benzofuran diferulic acid. Therefore, ferulic acid esterase A from *Aspergillus niger* contributes to total plant cell wall degradation by cleaving at least one ester bond from the diferulate cross-links that exist between wall polymers but does not always release the free acid product.

Keywords: esterase; ferulic acid; dehydrodiferulic acids; cross-links; plant cell walls.

Cinnamoyl esterases, a subclass of the carboxylic ester hydrolases (EC 3.1.1.1), hydrolyse the ester bond between hydroxycinnamic acids and sugars present in plant cell walls and have been purified and characterized from many micro-organisms [1]. They act synergistically with xylanases and pectinases to digest plant cell walls and facilitate the access of hydrolases to the backbone of wall polymers [2]. Ferulic acid esterase A (FAEA) from *Aspergillus niger* [3] is able to cleave specifically the (1→5) ester bond between ferulic acid and arabinose and shows high specificity of hydrolysis for a range of synthetic methyl esters of phenyl alkanoic acids [4]. The rate of reaction increases markedly when the substrates are small soluble feruloylated oligosaccharides derived from plant cell walls [5,6]. The rate of catalysis and apparent affinity of FAEA are influenced by the substituents on the aromatic ring and by the type, position of attachment and number of sugar moieties [1]. Although the catalytic properties of FAEA on a broad range of substrates have been well investigated, the topology of the active site has not yet been described. Based on a deduced sequence for FAEA [7] and chemical modification studies (F. O. Aliwan and G. Williamson, unpublished results), it appears that the enzyme active site contains a nucleophilic serine residue, and that a tryptophan residue and carboxylic groups are involved in substrate binding.

Peroxidase/H_2O_2 mediated oxidation of *trans*-ferulic acid esterified to the main polymers in primary plant cell walls can form several ferulic acid dehydrodimers (8-5-, 8-*O*-4-, 5-5- and 8-8-) [8], which have now been identified and quantified in several plant cell walls [8–12]. Ferulate dimers have been shown to covalently cross-link cell wall polymers [13–19] influencing physical and mechanical properties of the plant cell wall [20,21] and contributing to cell wall indigestibility, probably by limiting the accessibility of main chain-degrading enzymes to the structural polysaccharides [22,23].

It has been shown that some microbial esterases are able to release diferulic acids from plant cell walls. FAEA from *A. niger* is able to release 5-5-diferulic acid from presolubilized barley, wheat bran and sugar beet pulp [24,25]. The activity is increased in combination with xylanases. The esterase is also able to release some 8-*O*-4-diferulic acid from pretreated wheat bran [25]. We hypothesize that hydrolysis of dehydrodiferulates by esterases could occur either through a single association of enzyme and substrate where both ester bonds in the diester are hydrolysed before the enzyme and free acid product dissociate (Scheme 1):

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Abbreviations: FAEA, ferulic acid esterase A.

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$$E + DS \rightleftarrows E{-}DS \underset{EtOH}{\searrow} E{-}DS' \rightarrow E{-}MS \underset{EtOH}{\searrow} E{-}MS' \rightarrow E{-}diFA \rightarrow E + diFA$$

Scheme 1

or in two discrete steps where the monoester product of the first reaction is released and becomes a substrate for a second reaction (Scheme 2):

$$E + DS \rightleftarrows E{-}DS \underset{EtOH}{\searrow} E{-}DS' \rightarrow E + MS \rightarrow E{-}MS \underset{EtOH}{\searrow} E{-}MS' \rightarrow E + diFA$$

Scheme 2

where E is the free enzyme, DS is the dehydrodimer diester substrate, MS is the dehydrodimer monoester product, E–DS and E–MS are the dehydrodimer diester and dehydrodimer monoester enzyme-substrate complexes, E–DS′ and E–MS′ are the corresponding acyl-enzymes and diFA is the free acid product, dehydrodiferulic acid.

The release of dimers by esterases has been studied in large complex substrates such as whole and solubilized plant cell walls, which do not allow for the estimation of kinetic constants to determine the specificity of the enzyme. The development of chemical and enzymic procedures to synthesize methyl and ethyl esterified dehydrodiferulates [8,26] has provided us with a new tool to examine the specificity of esterases for hydrolysing dehydrodiferulates present in plant cell walls. In this paper, we have determined the catalytic properties of the FAEA from *A. niger* on a range of synthetic ethyl diferulate esters (5-5-, 8-*O*-4- and 8-5-benzofuran) and two oligosaccharide 5-5-diferulates isolated from maize bran in order to enhance our understanding of the catalytic preferences of FAEA. We have also investigated whether the hydrolysis of both ester bonds from the diester substrates occurs through one or two separate reactions. On the basis of our results, we propose that FAEA from *A. niger* contributes to the total degradation of plant cell walls by cleaving the diferulic cross-links between cell wall polymers.

MATERIALS AND METHODS

Source of enzymes

The cinnamoyl esterase FAEA was purified from culture supernatants of *A. niger* grown on oat spelts xylan according to a published procedure [3].

Preparation of substrates

Ethyl ferulate was prepared using ethanolic HCl prepared according to the method of Fieser & Fieser [27]. Diethyl 8-5-benzofuran diferulate was synthesized using peroxidase-H_2O_2 and purified by flash chromatography [26]. Diethyl 5-5-diferulate and diethyl 8-*O*-4-diferulate, were synthesized according to the methods of Richitzenhain [28] and Ralph *et al*. [8], respectively, and purified by reverse-phase preparative chromatography [29]. 5-*O*-(*trans*-feruloyl)-L-Ara*f*) (Ara-ferulate) and (*O*-β-D-Xyl*p*-(1→2)-[(5-*O*-(*trans*-feruloyl)-L-Ara*f*] (Xyl-Ara-ferulate) were isolated from maize bran by acid hydrolysis and gel filtration chromatography [30]. (*E*,*E*)-4,4′-Dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid, bis(L-Araf-5-*O*-yl) ester) (Ara-5-5-Ara) and ((*E*,*E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid L-Arg*p*-*O*-yl ester, β-D-Xyl*p*-(1 → 2)-L-Ara-5-*O*-yl ester) (Xyl-Ara-5-5-Ara) were also isolated from maize bran using mild acid hydrolysis [31] and purified further by preparative HPLC (see below). All other chemicals were of analytical or HPLC-grade purity.

Solubility of the substrates

As the diethyl diferulate substrates are poorly soluble in Mops buffer, dimethylsulfoxide was added to the incubation mixture to increase their solubility. The final concentration of organic solvent to be used for enzyme incubations was determined on the basis of the residual activity of FAEA at increasing concentrations of dimethylsulfoxide in 100 mM Mops (pH 6.1) using ethyl ferulate as substrate and on the basis of substrate solubility. It was found that at 20% (v/v) dimethylsulfoxide in buffer, the activity of FAEA on ethyl ferulate was approximately 70% of that in buffer alone. Substrates (0.05–0.5 mM concentration range) were incubated in 20% (v/v) dimethylsulfoxide/100 mM Mops buffer (pH 6.1). After incubation for 15 min at 37 °C, incubation mixtures were centrifuged (13 000 ***g***, 10 min), filtered (0.2 μm) and samples were immediately quantified by HPLC. Subsequently, incubation mixtures were allowed to stand at room temperature for several hours, centrifuged (13 000 ***g***, 10 min), filtered (0.2 μm) and reanalysed by HPLC. The maximum solubility of the substrates in 20% dimethylsulfoxide was: 5-5-diester, 0.28 mM; 8-5-benzofuran diester, 0.06 mM and 8-*O*-4-diester 0.2 mM. The solubility of the substrates tested was not increased significantly at concentrations of dimethylsulfoxide above 20% but the activity of the enzyme was decreased sharply by dimethylsulfoxide concentrations > 20% (50% residual activity in 30% dimethylsulfoxide; no activity detected in 50% dimethylsulfoxide). The effect of dimethylsulfoxide on the catalytic properties of FAEA was determined by incubating the enzyme with a range of concentrations of ethyl ferulate in 2.7% dimethylsulfoxide and 20% dimethylsulfoxide in 100 mM Mops, pH 6.1. The kinetic constants for the hydrolysis of ethyl ferulate by FAEA were: k_{cat} 65.3 ± 0.7 s^{-1}, K_m 0.69 ± 0.02 mM in 2.7% dimethylsulfoxide; k_{cat} 85.6 ± 0.2 s^{-1}, K_m 3.4 ± 0.1 mM in 20% dimethylsulfoxide. It appears that the organic solvent decreases the interaction between enzyme and substrate, as the K_m value obtained in 20% dimethylsulfoxide is five times higher that obtained in 2.7%.

Enzyme assays and kinetics

The activity of FAEA was assayed using ethyl ferulate, a range of synthetic diferulates, ethyl and diethyl esters of 5-5-, 8-*O*-4- and 8-5-benzofuran diferulic acids, and two 5-5-dimers isolated from maize bran where the dimers are esterified to sugars. For determination of kinetic parameters, incubation mixtures contained a range of concentrations (10–18, in duplicate) of the esterified substrate ([numbers in bold refer to Fig. 1; ethyl ferulate 5–1500 μM; 5-5-diester **1** 5–250 μM; 8-5-benzofuran diester **3** 5-50 μM; 8-*O*-4-diester **5** 5–150 μM; 5-5-monoester **2** 5–1500 μM; 8-*O*-4-monoester **6** 10–200 μM; Ara-ferulate 5–300 μM; Xyl-Ara-ferulate 5–300 μM; Ara-5-5-Ara 5–150 μM; Xyl-Ara-5-5-Ara 5–250 μM; for most of the synthetic diesterified substrates the upper limit of concentration was determined by the solubility) in 20% (v/v) dimethylsulfoxide in buffer (100 mM Mops, final pH 6.1 or 100 mM sodium citrate, final pH 3.2) and an amount of FAEA (8.3 ng to 13.3 μg per assay) that gave less than 5% conversion of substrate to product and was always in the linear part of the reaction curve. Incubations (0.5 mL final volume) were performed at 37 °C for 10–15 min and were terminated by the addition of acetic acid (0.2 mL, final pH < 2.5). The release of product was monitored by HPLC/diode array using the method described by Waldron *et al*. [32] with detection by absorbance at 280 nm and 325 nm. The response factors

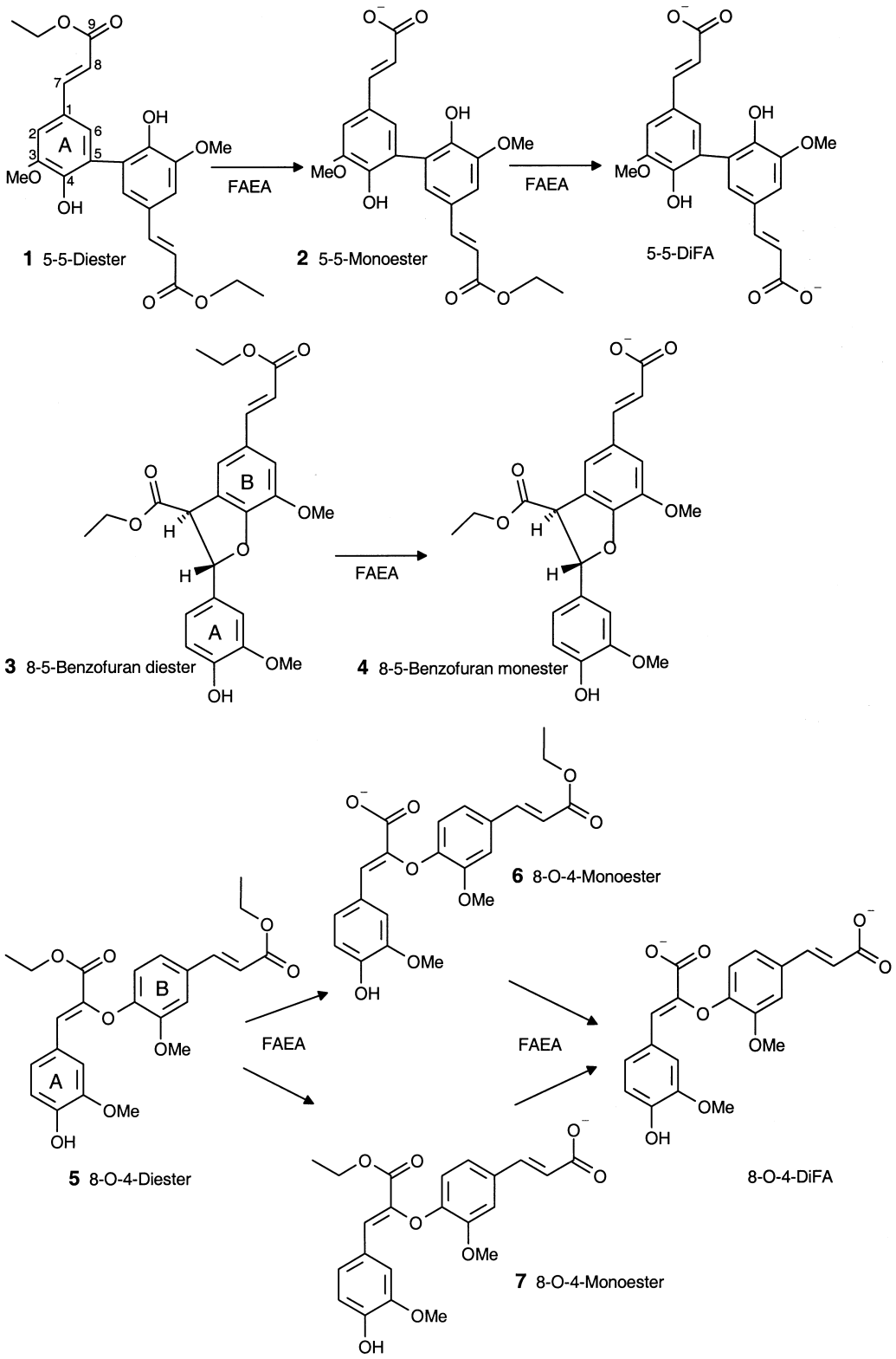


Fig. 1. Structures of the synthetic ethyl diesterified substrates and their monoesterified and free acid products after hydrolysis by FAEA from *A. niger*. The numbers in bold preceding the compound name are used to aid identification throughout in the text, tables and figures.

for the reaction products, monoesters and free acids, were calculated from appropriate solutions of purified synthetic compounds (purity of the compounds by NMR exceeded 95%). All enzyme assays were performed in duplicate. In parallel, blanks containing substrate plus enzyme in 20% dimethylsulfoxide in buffer were incubated with acetic acid as controls. The kinetic constants K_m and k_{cat} were estimated using a nonlinear regression method (ENZFITTER, Biosoft, Cambridge, UK) that also gives an estimate of the standard error of each parameter [33].

Table 1. Absorption maxima and absorption coefficients in ethanol for the ethyl-esterified diferulates 1–6 (see Fig. 1). The values shown are the mean and standard deviation of at least three determinations.

Compound	λ_{max} (nm)	λ_{min} (nm)	$\varepsilon_{\lambda max}$ ($M^{-1}\cdot cm^{-1}$)	$\varepsilon_{\lambda 280}$ ($M^{-1}\cdot cm^{-1}$)
5-5-Diester **1**	335.0	280.0	39 500 ± 2000	21 000 ± 1000
8-5-Benzofuran diester **3**	325.0	266.0	27 000 ± 1000	12 000 ± 1000
8-*O*-4-Diester **5**	329.0	261.0	32 000 ± 2000	20 000 ± 1000
5-5-Monoester **2**	329.0	276.0	41 000 ± 2000	25 000 ± 1000
8-5-Benzofuran monoester **4**	321.0	264.0	21 000 ± 1000	12 000 ± 1000
8-*O*-4-Monoester **6**	327.0	260.0	22 000 ± 2000	16 000 ± 2000

Preparation of monoesterified diferulates

Several milligrams of ethyl diester were incubated with FAEA (1 μg) in 20% (v/v) dimethylsulfoxide in 100 mM Mops (final volume 500 mL) for an extended period of time. Monoester formation was monitored by analytical HPLC and the reaction terminated by acidifying with acetic acid to pH < 2.5. The products were extracted with ethyl acetate (3 vol.), after which the extracts were evaporated to dryness, re-dissolved in 50% (v/v) aqueous methanol, and filtered (0.2 μm) prior to purification. The monoesters were purified by reverse-phase preparative chromatography [28]. Fractions containing the monoester were collected and pooled. Analytical HPLC showed single peaks for all the purified fractions.

Characterization of monoesterified products

The absorption properties, pK_a, molecular mass and structure of the monoesterified products formed were determined. The structures for all the diesterified substrates, monoesterified products and free acids are depicted in Fig. 1.

Absorption properties

The absorption coefficients for each dimer (diesters and monoesters) were obtained from the corresponding ethanolic solutions of the purified compounds [34] and are presented in Table 1.

Determination of pK_a

The pK_a value for the monoesterified ethyl 5-5-diferulate **2** was determined by UV/visible spectrophotometry [35]. The monoester was prepared in 20% (v/v) dimethylsulfoxide in a selection of buffers at low and constant ionic strength (0.01 M) ranging from pH 2.3–7.12. The wavelength at which the two pure species differed most in absorbance was 330 nm. Spectra were recorded at 20 °C.

Liquid chromatography-mass spectometry

Samples were separated [30] using a Philips PU 4100 HPLC (Cambridge, UK). Spectra were acquired using either a Micromass Platform (Manchester, UK) equipped with a conventional electrospray/atmospheric pressure chemical ionization ion source or a Micromass Quattro II mass spectrometer equipped with a Z-spray™ electrospray/atmospheric pressure chemical ionization ion source. Mass spectra were acquired continuously at unit mass resolution over a mass range of 120–2000 Da, at a scan rate of 3 s with an interscan delay of 0.1 s under the following conditions: probe temperature, 350 °C; source block temperature, 120 °C; corona voltage, 3.9 kV; cone voltage, 18 V. 5-5-Monoester **2** gives a protonated molecular ion $[M + H]^+$ at *m/z* 415 and other protonated molecular ions at *m/z* 371 (50), 325 (100); 8-5-benzofuran monoester **4** ion mass at *m/z* is 415 $[M + H]^+$, significant ions at *m/z*: 371 (56), 351 (100), 325 (60); 8-*O*-4-monoester **6** ion mass at *m/z* is 415 $[M + H]^+$, significant ions at *m/z*: 397 (100), 371 (42), 351 (57), 325 (44), 291 (24); 8-*O*-4 monoester **7** ion mass at *m/z* is 415 $[M + H]^+$, significant ions at *m/z*: 369 (100), 325 (35), 291 (37).

NMR spectroscopy

NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). Samples were dissolved in 0.6 mL of d_4-methanol (**1** and **2**) or d_6-acetone (**3**–**7**) in 5-mm NMR tubes and spectra were measured at 27 °C. Chemical shifts are reported with reference to tetramethylsilane using the methyl signal of the solvent as intermediate reference (methanol ^{1}H 3.30 p.p.m., ^{13}C 49.0 p.p.m.; acetone ^{1}H 2.04 p.p.m., ^{13}C 29.8 p.p.m.). The ^{1}H and ^{13}C chemical shifts for the ethyl esterified diferulates are given in Tables 2 and 3.

5-5-Diester **1** assignments are based on those published for the 5-5-dehydrodiferulic acid [8]. Both molecules have a plane of symmetry so that the two halves of each dimer, linked by the 5-5-bond, have identical chemical shifts. Compared with the

Table 2. Chemical shifts (p.p.m.) of the ^{1}H-resonances for the ethyl-esterified diferulates 1–7 (see Fig. 1).

Atom	1[a]	2[a]	3[b]	4[b]	5[b]	6[b]	7[b]
MeCH_2O	–	–	1.25	–	1.25	–	1.22
MeCH_2O	1.30	1.30	1.30	1.30	1.30	1.30	–
A3-OMe	–	–	3.80	3.80	3.75	3.75	3.75
B3-OMe	3.93	3.95	3.90	3.90	4.00	4.00	4.00
Me*CH_2*O	4.21	4.22	4.20[c]	–	4.20	4.20	4.20
Me*CH_2*O	–	–	4.30[d]	4.30	–	–	–
A8-H	6.36	6.37, 6.33	4.45	4.45	–	–	
A7-H	7.63	7.62	6.05	6.05	7.37	7.41	7.37
A2-H	7.19	7.21	7.10	7.10	7.50	7.51	7.50
A5-H	–	–	6.80	6.80	6.81	6.85	6.81
A6-H	7.10	7.08	6.90	6.90	7.21	7.22	7.22
B8-H	–	–	6.40	6.40	6.45	6.45	6.44
B7-H	–	–	7.60	7.60	7.55	7.56	7.55
B2-H	–	–	7.34	7.34	7.45	7.45	7.45
B5-H	–	–	–	–	6.79	6.80	6.79
B6-H	–	–	7.29	7.29	7.12	7.12	7.11

[a] In d_4-methanol; [b] in d_6-acetone; [c] Me*CH_2*O-B9; [d] Me*CH_2*O-A9.

Table 3. Chemical shifts (p.p.m.) of the ^{13}C resonances for the ethyl-esterified diferulates 1–7 (see Fig. 1).

Atom	1[a]	2[a]	3[b]	4[b]	5[b]	6[b]
MeCH_2O	14.65	14.64	14.49[c]	14.48	14.47	–
MeCH_2O	–	–	14.64[d]	–	14.59	14.60
A3-OMe	56.68	56.73	56.33	56.33	55.94	55.92
B3-OMe	–	–	56.51	56.51	56.52	56.49
Me*CH_2O*	61.41	61.44	62.21[e]	62.21	61.72	–
Me*CH_2O*	–	–	60.57[f]	–	60.61	60.60
A1	126.74	126.95, 127.00	132.10	132.11	125.19	125.31
A2	110.05	110.22	110.77	110.78	113.85	113.82
A3	149.96	148.00, 149.62	148.56	148.54	148.73	148.29
A4	148.85	147.15, 147.93	148.00	147.90	149.58	149.41
A5	126.54	126.41, 126.31	115.84	115.77	116.03	115.93
A6	126.64	126.48	120.17	120.18	126.04	126.04
A7	146.85	146.71, 147.00	88.35	88.32	128.04	128.43
A8	115.60	115.89, 116.16	56.09	56.07	138.41	138.33
A9	169.36	169.30, 170.95	171.11	171.11	163.79	164.58
B1	–	–	129.47	129.53	130.18	130.04
B2	–	–	113.34	113.41	112.42	112.36
B3	–	–	145.83	145.82	150.17	150.23
B4	–	–	150.99	150.96	148.86	148.96
B5	–	–	127.42	127.42	114.54	114.39
B6	–	–	118.92	118.85	122.94	122.97
B7	–	–	145.23	145.56	144.83	144.87
B8	–	–	116.71	116.77	117.57	117.47
B9	–	–	167.28	168.02	167.20	167.20

[a] In d_4-methanol; [b] in d_6-acetone; [c] *Me*CH_2O-A9; [d] *Me*CH_2O-B9; [e] Me*CH_2O*-B9; [f] Me*CH_2O*-A9.

diacid, the diester shows two extra proton resonances at 1.30 p.p.m. and 4.21 p.p.m. corresponding to the *Me*CH_2O and Me*CH_2O* protons of the two ethyl groups. Proton resonances are similar in the 5-5-monoester **2** although the olefinic and aromatic proton shifts are affected by removal of one of the ethyl groups. The effect is greatest for 8-H (the numbering scheme is given in Fig. 1) which appears as a pair of doublets (6.33, 6.37 p.p.m.) in **2** instead of a single doublet as in **1**. Integration shows that **2** only contains one ethyl group. Most of the carbon atoms show doubled chemical shifts in the monoesterified compound due to the presence of one esterified and one nonesterified carboxylic group and the consequent loss of symmetry. The splitting was greatest for carbons 9, 4 and 3.

Proton and carbon assignments for diethyl 8-5-coupled dehydrodiferulate **3** were identical to those reported for the diethyl dimer by Ralph *et al*. [8]. Compound **3** shows two separate ^1H-chemical shifts for the two *Me*CH_2O groups (1.25 and 1.30 p.p.m.) and for the two Me*CH_2O* groups (Me*CH_2O*-B9, 4.20 p.p.m. and Me*CH_2O*-A9, 4.30 p.p.m.) corresponding to the two ethyl groups esterifying the two different carboxylic groups (rings A and B). 8-5-Benzofuran monoester **4** ^1H-resonances are almost identical to those of **3** except for the presence of only one *Me*CH_2O group (1.30 p.p.m.) and one Me*CH_2O* group (4.30 p.p.m.) in **4.** The Me*CH_2O* group has the same chemical shift as the one linked to the A9 in **3**. This result indicates that the monoesterified product **4** has the ethyl group esterified to the carboxylic group associated with ring A. The loss of the ethyl group esterified to ring B is further confirmed by ^{13}C-NMR as **4** does not show the carbon resonances at 14.64 p.p.m. and 60.57 p.p.m. that correspond to *Me*CH_2O-B9 and Me*CH_2O*-B9, respectively [8] in **3**. The presence of a nonesterified carboxylic group in ring B of **4** increases the carbon shifts for the atoms B7 (0.3 p.p.m.) and B9 (0.74 p.p.m.) in comparison with **3** whereas the shifts of A9, A8 and A7 are essentially unaltered. Compound **4** is thus the 8-5 benzofuran monoester depicted in Fig. 1.

The 8-*O*-4-diester **5** assignments are based on those published for the ethyl/methyl diester of 8-*O*-4-diferulate [8]. Compound **5** shows two separate ^1H-resonances corresponding to the two *Me*CH_2O groups of each phenolic ring (1.25 and 1.30 p.p.m.). The Me*CH_2O* protons give only one multiple peak at 4.2 p.p.m. Monoester **6** shows only the proton resonance at 1.30 p.p.m. whereas monoester **7** shows only the resonance at 1.22 p.p.m., indicating that these two structures correspond to the two possible monoesters that can be formed by cleaving each one of the ester bonds. Only **6** was available in sufficient quantity for ^{13}C-NMR. The diester has two separate carbon resonances for both *Me*CH_2O and Me*CH_2O* for the two ethyl groups whereas **6** has only one pair of chemical shifts corresponding to one ethyl group (14.60 and 60.60 p.p.m.). For confirmation of the structure we compared the carbon spectrum of **6** with those of the diester, **5,** and the 8-*O*-4-dehydrodiferulic acid [8]. The loss of one ethyl group from **5** to **6** changes certain carbon chemical shifts (assigned to A9, A7 and A3 of **5**) giving values which are almost identical to those found for the corresponding carbons of 8-*O*-4-diferulic acid. However, the shifts of the carbons assigned to the B-ring of the diester (notably B9) are unchanged in **6**. These results indicate that the ester bond cleaved by FAEA is the one located in ring A.

RESULTS

Hydrolysis of synthetic diferulate ethyl esters (5-5-, 8-*O*-4- and 8-5-benzofuran) by FAEA

Analysis of reaction mixtures obtained after incubation of FAEA with any of the three diethyl diester substrates tested indicated that products had been formed. For example,

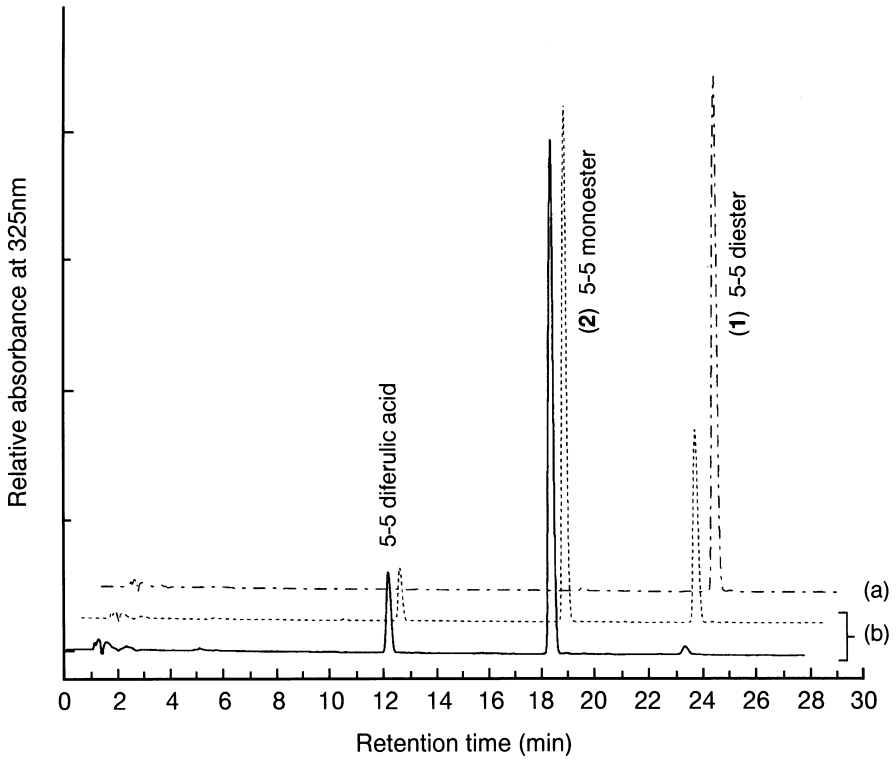


Fig. 2. HPLC analysis of the hydrolysis of a diesterified diferulate substrate by FAEA from *A. niger*. Substrate and products were separated on a C_{18} column using a gradient of increasing organic concentration (MeOH, ACN) at a flow rate of 1 mL·min^{-1}. (a) HPLC elution profile of diethyl 5-5 dehydrodiferulate; (b) HPLC traces showing the formation of the reaction products, 5-5 monoester and 5-5 diferulic acid, after incubation with FAEA from *A. niger*. Detection at 325 nm. The lines have been offset in the *x* and *y* axes.

incubation of 5-5-diethyl diester with FAEA and analysis of products by HPLC gave rise to three separate peaks (Fig. 2), which were identified as substrate **1,** 5-5-monoester **2** and 5-5-diferulic acid. Under the conditions of our assay, FAEA from *A. niger* was active on the three ethyl-diesterified substrates tested and the initial product was always a monoester as shown by LC-MS and NMR (see characterization of monoesters in Materials and methods). The conditions were set up to study separately the hydrolysis of diester to monoester and the hydrolysis of monoester to free acid. The catalytic efficiency (k_{cat}/K_m) and where possible individual k_{cat} and K_m values were estimated. The results of the kinetic analysis on the ethyl esterified dimers are presented in Table 4.

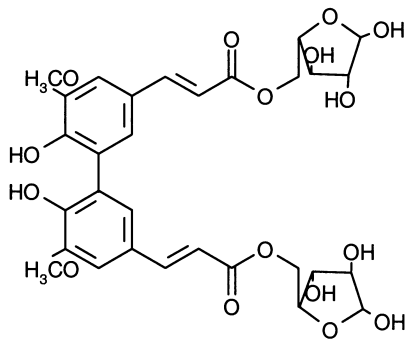
FAEA showed the highest catalytic efficiency (k_{cat}/K_m) for the hydrolysis of the 5-5-diesterified substrate **1** to form the 5-5-monoester **2**. The second most efficient reaction was the hydrolysis of the ester bond specifically on ring B of 8-5-benzofuran diester **3** to form 8-5-benzofuran monoester **4**. The enzyme was not active on the second ester bond and did not form 8-5-benzofuran diferulic acid even after an extended period of incubation with large amounts of enzyme. Hydrolysis of the 8-*O*-4-diester by FAEA, formed two separate products identified as monoester **6** and monoester **7**. Although the 8-*O*-4-diester and the monoester **6** were poor substrates for FAEA ($k_{cat}/K_m \leq 0.1$ s·mM^{-1}), the esterase was able to form 8-*O*-4-diferulic acid after a extended period of incubation. We were not able to estimate K_m and k_{cat} for the 8-*O*-4 monoester **7** as there was insufficient product available to calculate a response factor for this compound.

The catalytic efficiency of FAEA in hydrolysing 5-5- and 8-5-diesterified dimers to form the monoesterified products was higher than the efficiency of the esterase in releasing

Table 4. Steady-state kinetic parameters for FAEA (*A. niger*) hydrolysing a range of synthetic and natural esterified ferulates and diferulates.

Substrate	Product	(k_{cat} s^{-1})	(K_m mM)	(k_{cat}/K_m s^{-1}·mM^{-1})
Ethyl ferulate [a]	Ferulic acid	85.6 ± 0.2	3.4 ± 0.1	25.0
5-5-Diester **1** [a]	5-5-Monoester **2**	137.0 ± 11.0	0.74 ± 0.1	185.0
5-5-Monoester **2** [a]	5-5-Diferulic acid	32.4 ± 0.9	2.0 ± 0.1	16.0
8-5-Benzofuran diester **3** [a]	8-5-Benzofuran monoester **4**	–	–	99.4
8-*O*-4-Diester **5** [a]	8–O-4-Monoester **6**	–	–	0.10
8–*O*-4-Monoester **6** [a]	8-*O*-4-Diferulic acid	–	–	0.002
Ara-ferulate [a]	Ferulic acid	132.0 ± 12.0	0.27 ± 0.03	489.0
Ara-5-5-Ara [a]	5-5-Ara	140.0 ± 0.2	0.08 ± 0.01	1750.0
Xyl-Ara-ferulate [a]	Ferulic acid	126.0 ± 12.0	0.11 ± 0.02	1145.0
Xyl-Ara-5-5-Ara [a]	5-5-Ara	57.0 ± 0.2	0.12 ± 0.01	475.0
Xyl-Ara-5-5-Ara [a]	Xyl-Ara-5-5	40.0 ± 2.5	0.07 ± 0.01	571.0
Ethyl Ferulate [b]	Ferulic acid	7.1 ± 0.3	1.42 ± 0.12	5.0
5-5-Diester **1** [b]	5-5-Monoester	21.0 ± 3.5	0.14 ± 0.04	150.0
5-5-Monoester **2** [b]	5-5-Diferulic acid	1.8 ± 0.8	0.08 ± 0.02	22.5

[a] In 20% (v/v) dimethylsulfoxide in 100 mM Mops, pH 6.1; [b] in 20% (v/v) dimethylsulfoxide in 100 mM sodium citrate, pH 3.2.



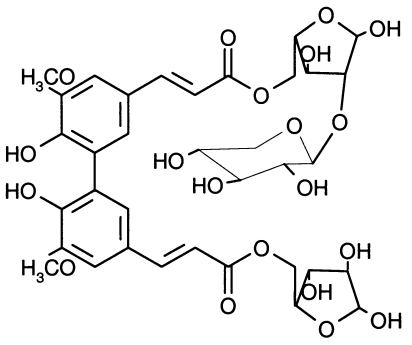


Fig. 3. Structures of the two natural diferuloylated oligosaccharides isolated from maize bran, Ara-5-5-Ara and Xyl-Ara-5-5-Ara.

ferulic acid from the esterified monomer. The K_m estimated for the 5-5-dimer is about fivefold lower than that for the ethyl ferulate indicating a higher affinity of FAEA for the dimer than for the monomer.

The monoester derived from the hydrolysis of 5-5-diester is also a substrate for FAEA although the efficiency of FAEA in hydrolysing the monoester to form 5-5-diferulic acid is about 10-fold lower than for the diester. Both the catalytic rate and the affinity were affected; k_{cat} was decreased by $\approx$ fourfold whereas K_m was increased by almost threefold. Besides possible conformational changes derived from the loss of symmetry in the 5-5-monoester, the presence of a negatively charged carboxylic group in the monoesterified substrate may be responsible for the reduction in the catalytic efficiency by affecting the interaction between substrate and enzyme. A similar reduction in efficiency was observed between the 8-*O*-4-diester **5** and the 8-*O*-4-monoester **6**.

Activity of FAEA on the 5-5-monoester: effect of substrate ionization

To investigate the possible influence of an ionized carboxylic group on the interaction between the monoesterified substrate and the catalytic site of the esterase, we examined the activity of FAEA on ethyl ferulate, 5-5-diester **1** and 5-5-monoester **2** in 20% dimethylsulfoxide (v/v) at pH 3.2 using 100 mM sodium citrate buffer (Table 4). At this pH, the carboxylic group of the 5-5-monoester **2** is present in the nonionized form whereas at pH 6.1 the molecule is present in its dissociated form (pK_a 4.67 $\pm$ 0.05). The possibility of irreversible changes in the enzyme, due to exposure to acidic conditions, was examined by analysing the recovery of the activity at pH 6.1 after incubation for 15 min at low pH. No significant losses of activity were observed. Under acidic conditions, the catalytic rate for the three substrates fell approximately 10-fold. The K_m values estimated for the monomer, the dimeric diester and the dimeric monoester at pH 3.2 were lower by approximately twofold, fivefold and 25-fold, respectively, indicating that the enzyme has a higher apparent affinity under these conditions. The increase in the affinity was much greater for the monoesterified substrate than for the diester or for ethyl ferulate.

Specificity of FAEA for natural diferulate oligosaccharides: effect of sugar esterification

We examined the catalytic properties of FAEA on two 5-5-diferulate oligosaccharides isolated from maize bran (Fig. 3) in 20% (v/v) dimethylsulfoxide/100 mM Mops, pH 6.1. The kinetic parameters were compared to those obtained for the activity of FAEA on the ferulate oligosaccharides, Ara-ferulate and Xyl-Ara-ferulate, under the same incubation conditions (Table 4). The presence of sugars esterified to ferulic and to 5-5-diferulic acid increases greatly the catalytic efficiency of FAEA. Ara-5-5-Ara was the best substrate for FAEA. Esterification of both carboxylic groups of the 5-5-dimer with arabinofuranoside reduces the K_m by 10-fold compared to the diethyl ester whereas the catalytic rates were similar, and, as a result, the catalytic efficiency was increased by one order of magnitude. The apparent affinity was slightly lower for the ferulated oligosaccharide, Ara-ferulate. When xylose is attached to the C-2 position of arabinose, the enzyme was more efficient hydrolysing the monomeric substrate, Xyl-Ara-ferulate, than the diesterified substrate, Xyl-Ara-5-5-Ara.

For both diesterified substrates, a sugar-monoesterified intermediate was also the first product of the reaction, although in extended incubations with FAEA, 5-5-diferulic acid was also detected.

DISCUSSION

FAEA from *A. niger* is able to hydrolyse either of the ester bonds in 5-5- and 8-*O*-4-dehydrodiferulates to form monoesters, and subsequently to hydrolyse the monoesters to form the corresponding free diferulic acids. The enzyme is also able to specifically cleave one of the ester bonds in 8-5-benzofuran diester forming one of the possible monoesters. These results are in agreement with the ability of the esterase to release 5-5- and 8-*O*-4-diferulic acids, but not 8-5-benzofuran diferulic acid, from solubilized plant cell wall materials [24,25].

The differences observed in the specificity of FAEA for the three types of dehydrodimer can be attributed to differences in their structure. It has been shown that the specificity of microbial esterases differs markedly across a series of synthetic substrates. The distance between the phenolic ring and the ester bond and the number and position of methoxy and hydroxy substitutions on the benzene ring are critical in determining the catalytic efficiency of FAEA [4]. The inability of FAEA to hydrolyse the ester bond on ring A of the 8-5-benzofuran dimer may be due to the 7-O-4 ether linkage which alters the olefinic part of the molecule. This may affect the distance between this ester bond and the aromatic ring A, and hence prevent catalysis. Differences in conformation, intramolecular bond lengths and angles, and exposure of reactive groups and of ester bonds for each particular type of coupled ferulate have a clear effect on esterase activity.

We have shown that FAEA is highly efficient at hydrolysing

diesterified dehydrodiferulates. The first product of the hydrolysis, always a monoester, is also a substrate for FAEA but the specificity is lower than for the diester, i.e. the kinetic data indicate that the enzyme *in vivo* is more efficient at breaking the cross-links between polymers than at releasing free diferulic acid. Therefore, release of diferulic acids from plant cell wall materials is not a good indicator of cross-link cleavage. In order to study the breaking of cross-links in plant cell walls, novel methods need to be developed, possibly involving *in situ* detection of dehydrodimers and putative monoester products in intact plant cell walls.

It has been shown that diferulate cross-links between the main polymers in plant cell walls reduce cell wall digestibility by limiting the accessibility of hydrolytic enzymes [22,23]. We propose that FAEA from *A. niger* contributes to plant cell wall degradation by cleaving the most abundant diferulate cross-links present in plant cell walls. It should be noted that FAEA exhibits highest specificity for hydrolysis of the 5-5-dimer which is not one of the most abundant dimers present in plant cell wall materials [8–12], but it is the only dehydrodimer for which there is direct evidence of involvement in cross-linking plant cell wall polysaccharides [18]. These results appear to indicate that *A. niger* preferentially cleaves 5-5-cross-links as an important event in degradation of complex cell wall polymers. However, it is more likely that there are other esterases whose specificities complement those of *A. niger* FAEA in breaking these cross-links, and which form part of the battery of hydrolytic enzymes required for complete degradation of complex polysaccharides.

Structural studies and the use of specific chemical inhibitors have indicated that hydrophobic binding sites are important components of the active sites in several esterases with diverse specificities (e.g. acetylcholinesterases, butyrylcholinesterases, lipases, cholesterol esterases, carboxyl esterases, proteases). For example, the crystal structure of acetylcholinesterase reveals that the active site of the enzyme lies near the bottom of a narrow gorge which contains 14 aromatic residues [36]. More recently it has been shown that a carboxylesterase from *Pseudomonas fluorescens* has an active-site cleft with an inside surface of the surrounding walls composed of mainly aliphatic side chains [37]. Chemical modification studies (F. O. Aliwan and G. Williamson, unpublished results) suggest that a single tryptophan residue and carboxylic group(s) are involved in the binding of substrate by FAEA. Our data appear to indicate that FAEA binds substrates more tightly at lower pH. There was some evidence that for the monoester substrate, neutralization of the negative charge on the carboxylic group (at low pH) further improved the interaction (based on K_m values) between the enzyme and the substrate. This may indicate that hydrophobic and/or carboxylic residues in FAEA are involved in catalysis, but, as K_m values are a function of all the rate constants for all the steps on the reaction pathway, we cannot discount the effect of pH on these rate constants.

The presence of organic solvent in the reaction medium influences the interaction between enzyme and substrate, which is due mainly to electrostatic and hydrophobic interactions. In the case of hydrophobic binding, the K_m value is usually higher in water-solvent mixtures compared to aqueous solutions [38]. The fivefold increase in the K_m of FAEA for ethyl ferulate in 20% dimethylsulfoxide suggests that hydrophobic interactions are involved in binding. As 5-5-, 8-*O*-4- and 8-5-benzofuran diferulic acids are more hydrophobic molecules than ferulic acid [39], the hydrophobicity of diferulate substrates probably also contributes to give the observed increase in specificity compared to ferulates.

The apparent affinity of feruloyl esterases for cinnamate substrates is greatly enhanced when the cinnamic acid is esterified to sugars compared to alkyl esters [1]. The type of sugars present, the length of the oligosaccharide and the position of the linkage between the primary and secondary sugars, all influence the release of ferulic acid by FAEA [5]. FAEA is more specific for 5-5-diferulate substrates derived from plant cell walls than for alkyl esters such as methyl and ethyl ferulate. The enzyme shows some specificity also for the oligosaccharide chain to which the 5-5 diferulate is linked. It was possible that for alkyl diester substrates, the release of monoester intermediates was due to a reduced affinity between FAEA and intermediate, whereas with sugar diesters, we may have expected the increased affinity to prevent dissociation of the enzyme–sugar monoester intermediate (Scheme 1). However, we have detected sugar monoesterified intermediates as a result of hydrolysis of sugar diester substrates, and this indicates that the presence of sugars does not prevent dissociation of the intermediate from the enzyme–substrate complex.

In conclusion, the hydrolysis of diferulates to free acids by FAEA from *A. niger* proceeds in two discrete steps involving dissociation of a monoester intermediate which is negatively charged at the pH of the reaction shown in Scheme 2.

The esterase is highly specific for hydrolysing one ester bond from 5-5- and 8-5-dehydrodiferulates which cross-link plant cell wall polymers and we propose that this esterase contributes to total degradation of plant cell walls by cleaving diferulate cross-links. Our results also provide some evidence for the involvement of carboxylic groups and hydrophobic interactions in the binding of substrates.

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